Isolation of the specific glomerular basement membrane antigen involved in Goodpasture syndrome

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ABSTRACT The antigen involved in the glomerulonephritis associated with antibodies to glomerular basement membrane (GBM) was purified from human GBM digested with highly purified clostridial collagenase. The purified nonreduced sample contained two components with closely similar mobilities on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. After reduction they moved as one, nonantigenic, component, corresponding to a molecular weight of 26,000. Immunologically identical aggregates of higher molecular weight (i.e., 48,000) were also identified in the crude digest. Reduction of such aggregates after purification released some protein with a molecular weight of 26,000, but a large proportion was insensitive to reduction. Seven patients with Goodpasture syndrome all had circulating anti-GBM antibodies directed only against the purified antigen.

Antibodies to glomerular basement membrane (GBM) are most commonly associated with the triad of glomerulonephritis, alveolar necrosis, and linear deposits of immunoglobulins along the basement membranes of glomerulus and lung (1). The typical clinical syndrome was described by Goodpasture (2) and is therefore often referred to as Goodpasture syndrome (GP). More recently, variant cases not having the typical clinical picture have been described (1). Nevertheless these cases also are referred to as GP (1). One major constituent of GBM is the type IV collagen molecules having nonfibrillar extensions in both NH₂- and COOH-terminals (3, 4). During the last few years, a number of other distinct basement membrane macromolecules — i.e., laminin (5), heparan sulfate proteoglycans (6), entactin (7), and 7S collagen (8) — have been isolated.

Several attempts have been made to characterize the basement membrane antigen involved in GP. In 1971 McIntosh and Griswold (9) suggested that the antigen is collagen in nature, on the basis of their finding that kidney sections treated with collagenase did not bind antibodies present in GP serum. On the other hand, Marquardt et al. (10) could show, using in vitro techniques, that the antigen was a noncollagenous glycoprotein since collagenase digestion did not abolish binding of antibodies from patients. In an assay designed by Mahieu et al. (11), binding of antibodies from patients with GP to antigens extracted from basement membranes could be inhibited by using a preparation of disaccharide-peptides from collagen. Other sets of findings are the observation that GP sera react neither with type IV collagen nor with laminin (12) and the contradictory observation that the antibodies are indeed directed to laminin and type IV procollagen (13). In previous studies (14) we have shown that antibodies from patients with GP react with antigen solubilized from GBM with collagenase but not with antigen solubilized with pepsin — i.e., type IV collagen. Furthermore, Hunt et al. (15) have isolated three antigenic fractions of noncollagenous origin by using affinity chromatography of collagenase digests of basement membrane on a column containing bound GP antibodies.

One problem in the diagnosis of anti-baseament membrane antibody disease is that circulating antibodies directed against noncollagenous basement membrane components are present in several forms of glomerulonephritis (16). Antibodies present in GP patients, however, react with a unique antigen (16) present in all basement membranes studied (including GBM and lung basement membrane), allowing specific diagnosis of GP by immunoassay.

MATERIALS AND METHODS

Patients, Diagnostic Criteria. All seven patients had rapidly progressive crescentic glomerulonephritis. One male patient had serious lung purpura with massive hemoptysis, while lung involvement was less pronounced in the others. Immunofluorescence staining of biopsy specimens showed the typical linear pattern of IgG deposition along the GBM in all patients and along lung basement membrane in the one patient with severe lung involvement. The presence of circulating anti-GBM antibodies was verified by ELISA.

Preparation of GBM. Human kidneys were obtained at autopsy performed within 16 hr after death and stored frozen until processed. The adaptation by Westberg and Michael (17) of the method of Spiro (18) and further modified as described (19) was used to prepare glomeruli and basement membrane. All preparative steps were done with ice-cold, aqueous wash solutions containing the protease inhibitors 4 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine-HCl, 25 mM 6-aminohexanoic acid, and 10 mM EDTA. Isolated glomeruli were sonicated to remove cellular material. The remaining insoluble basement membrane was lyophilized and stored dry.

Digestion with Collagenase. Collagenase ( Worthington CLS) was purified according to Lee-Own and Anderson (20) and assayed for remaining nonspecific protease by using [14C]acetylated hemoglobin (21). In the presence of the protease inhibitors listed above, omitting EDTA, no proteolytic activity could be demonstrated in the purified enzyme. By using a Potter–Elvehjem homogenizer, GBM was suspended at 10 mg/ml in 0.05 M Hepes, pH 7.5/0.01 M CaCl₂/0.05% NaN₃ containing the protease inhibitors listed above, omitting EDTA. Collagenase was added at a ratio to GBM of 1:50 (wt/wt) and digestion was performed at 37°C for 72 hr (22). The solution was centrifuged at 105,000 × g for 1 hr to remove all particulate matter and the supernatant was stored in the cold (4°C). To determine the kinetics of the digestion, samples were withdrawn at different time intervals.

Abbreviations: GBM, glomerular basement membrane; GP, Goodpasture syndrome; GP antibodies, antibodies present in patients with GP and directed against GBM; GP antigen, antigen reacting with antibodies from patients with GP.
ELISA. The assay for the GP antigen was essentially the ELISA already described (14, 16) with minor modifications. Coating was found to be most efficient in 6 M guanidine-HCl, possibly by the prevention of aggregation of antigen. Furthermore, inclusion of guanidine-HCl to make all solutions 0.6 M was found to increase the degree of inhibition and thereby the sensitivity of the assay.

Chromatography. The proteins solubilized from the basement membrane by collagenase digestion were fractionated on a column (2.0 × 140 cm) of Sephacryl S-200 (Pharmacia), eluted with 0.05 M Tris-HCl, pH 7.5, also containing 0.15 M NaCl, 0.001 M EDTA, and 0.05% NaN3; 6-ml fractions were collected. After analyses, those fractions containing GP antigen were pooled. The antigen was further purified by affinity chromatography on a column of Sepharose CL-4B in 0.05 M Tris-HCl, pH 8.0/0.5 M NaCl to which rabbit IgG directed against collagenase and rabbit IgG directed against particulate human GBM had been coupled with cyanoargent bromide (23). The material not bound to the affinity column was concentrated by ultrafiltration through a UM 2 filter (Amicon), dialyzed against 6 M guanidine-HCl/0.05 M Tris-HCl, pH 7.5, and chromatographed on a column (2.6 × 143 cm) of Sephacryl S-200 eluted with 6 M guanidine-HCl/0.05 M Tris-HCl, pH 7.5. The fractions (each 10 ml) containing GP antigen activity were pooled and dialyzed against 8 M urea/0.04 M sodium acetate, pH 4.8, and chromatographed on a column of CM-cellulose (Whatman, Maidstone, Kent, England) (1 × 10 cm) equilibrated in the same buffer. A linear gradient of 0-0.3 M NaCl was used for elution and 8-ml fractions were collected.

Electrophoresis. NaDodSO4/polyacrylamide gel electrophoresis was done as described by Laemmli (24), in 10%, 12%, 12.5%, 14%, or 16% gels. Gels were stained with Coomassie brilliant blue R-250 (Merck, Darmstadt, Federal Republic of Germany). Proteins in aliquots of the fractions were precipitated with 10 vol of ethanol containing 10 mM sodium acetate before electrophoresis. In some instances gels were stained with silver as described by Morrissey (25). Proteins separated on 12.5% NaDodSO4/polyacrylamide gel electrophoresis were blotted onto nitrocellulose paper (Bio-Rad) as described by Burnette (26). Bound proteins were detected by incubation of the papers with diluted (1:50) serum from a GP patient for 1 hr, followed by incubation with antihuman IgG labeled with horseradish peroxidase (Dakoppats, Copenhagen, Denmark) for 1 hr. Peroxidase activity was detected by using 3-amino-9-ethyl-carbazole as the substrate.

Modification of Antigens. The purified GP antigen was unfolded by reduction with dithiothreitol and alkylated with iodoacetic acid as described (16).

RESULTS AND DISCUSSION

Collagenase Digestion. The purified collagenase was used to specifically degrade the triple-helical parts of the GBM, thereby releasing the intact GP antigen. The digestion of the GBM was continued for 72 hr (22) to obtain a limit digest of the collagenous portions of the membrane. Under these conditions 70-80% of the membrane (wt/wt) was solubilized. Kinetic studies showed that solubilized GP antigen increased rapidly during the first 4 hr and then increased more slowly until 72 hr. It appeared that extended digestion did not destroy the released antigen, since even on prolonged digestion the GP antigen activity did not decrease.

Isolation of the GP Antigen. The proteins solubilized by the collagenase digestion were fractionated by gel chromatography on Sephacryl S-200 (Fig. 1). The chromatogram showed several peaks in the void volume (S-200-I), a major peak with a Kav of about 0.17 (S-200-II), and a mid peak with a Kav of 0.44 (S-200-III). More UV-absorbing material eluted late but contained no GP antigen. The major portion of the GP antigen eluted in the S-200-III peak but appeared not to be homogenous (Fig. 1). Additional GP antigen was eluted in the first and second peaks (S-200-I and -II), possibly a result of aggregation of components or in-complete degradation of the membrane. The first peak (S-200-I) contained various undefined basement membrane components as shown by strong reaction with an antisera raised in rabbits against intact GBM (not shown). The second peak, among other components, albumin derived from serum contaminant and the collagenase used for digestion (data not shown).

To obtain a better idea of the number of components present, samples of selected fractions were separated by electrophoresis on NaDodSO4/12.5% polyacrylamide gels (not shown). Fraction S-200-III contained major components with mobilities corresponding to molecular weights of about 48,000 and 26,000, possibly representing the GP antigen. These components were also present in fraction S-200-II.

To further purify the GP antigen the pooled fraction S-200-III was passed through an affinity column containing bound anti-collagenase antibodies and anti-GBM antibodies. The latter were raised in rabbits against particulate human GBM and did not react with the GP antigen. The anti-GBM antibody preparation in addition contained antibodies to plasma proteins, which constitute 1-2% of the isolated membrane (10). Such contaminating plasma proteins were, however, removed by the affinity column. The material not bound to the immunosorbent column contained 95% of the chromatographed GP antigen, while neither albumin nor collagenase could be detected by ELISA. Electrophoresis of this material on NaDodSO4/polyacrylamide gels (not shown) showed four components with apparent molecular weights of about 60,000, 48,000, 26,000, and 20,000. Since these components were well separated in the denaturing conditions of the NaDodSO4 electrophoresis, an attempt was made to further purify the antigen by gel chromatography under the dissociative conditions of 6 M guanidine-HCl. The material not bound to the affinity column was concentrated by ultrafiltration (UM 2) and subsequently dialyzed against 6 M guanidine-HCl/0.05 M Tris-HCl, pH 7.5. The proteins were then chromatographed on a column of Sephacryl S-200 eluted with the same solvent (Fig. 2). Two protein peaks plus UV-absorbing material eluting in the total volume could be seen. More than 96% of the GP antigen chromatographed in the second peak (II), while the remaining activity chromatog-
Fig. 2. Chromatography on Sephacryl S-200 of affinity-purified GP antigen under dissociating conditions—i.e., in 6 M guanidine-
HCl/0.05 M Tris-HCl, pH 7.5. The fractions were analyzed for their content of protein by absorbance at 280 nm and for content of GP antigen by ELISA. Proteins in aliquots of fractions, indicated by I and II, were electrophoresed on NaDodSO4/15% polyacrylamide gels under nonreducing conditions. Molecular weight markers, however, were reduced prior to electrophoresis.

graphed in the first (I). The proteins in the peaks were further identified by NaDodSO4/polyacrylamide gel electrophoresis. Peak II contained only one component, with an apparent molecular weight of 26,000, and this material was considered as pure GP antigen. The overall yield of the GP antigen represents about 10% of the proteins in the collagenase digest used for purification. The component present in peak I has a mobility corresponding to a molecular weight of 48,000. It was not further analyzed.

Characterization of the GP Antigen. The purity of the GP antigen was tested by chromatography on a column of CM-
cellulose equilibrated in 8 M urea/0.04 M sodium acetate, pH 4.8. Proteins were eluted with a salt gradient from 0–0.3 M NaCl (Fig. 3A). A small amount of UV-absorbing material did not bind to the column. This material contained no reactive antigen and no protein was detected on NaDodSO4/polyacrylamide gel electrophoresis. The major portion of the protein eluted with about 0.2 M NaCl. This peak showed some microheterogeneity, and the fractions eluted at higher ionic strength had a higher relative content of GP antigen.

NaDodSO4/polyacrylamide gel electrophoresis of nonreduced samples showed the presence of two components (Fig. 3B). The particular preparation shown also contained a minor component (less than 1%) with a somewhat higher mobility on NaDodSO4 electrophoresis [fractions 18 and 20 (Fig. 3B)]. Protein blotting using serum from a GP patient showed only one component (Fig. 3C), with a mobility corresponding to the slower-moving band. After reduction the two components had the same mobility (Fig. 3D). It is possible that the two components observed represent closely similar structures, perhaps originating from the globular portions of two types of type IV collagen chains, as is discussed below. Only one of the components appears to represent the antigen.

Relationship of Activity to Structure. Reduction and alkylation of the GP antigen in 6 M guanidine-HCl completely inhibited its reaction in ELISA (data not shown), demonstrating that the antigen is protein and contains intrachain disulfide bonds that are essential for the GP antigen activity. Supporting evidence is the absence of reactive antigen in immunoblots of samples reduced prior to NaDodSO4/polyacrylamide gel electrophoresis (data not shown).

Structure and Composition. To determine the molecular weight of the GP antigen, NaDodSO4/electrophoresis of reduced samples and reference proteins was performed on gels having different concentrations of acrylamide (10%, 12%, 14%, and 16%). A Ferguson plot (27) was constructed by plotting the relative migration distance for all proteins electrophoresed against the different concentrations of acrylamide (not shown). The line for the GP antigen extrapolated to the same point as the reference proteins, showing that the GP antigen behaved ideally in the system. Its molecular weight was calculated to be 26,000.

The amino acid composition of the GP antigen is shown in Table 1. The antigen contained only traces of hydroxyproline and no hydroxylysine. Furthermore, its content of glycine is only half that of collagenous proteins. It is therefore highly unlikely that the GP antigen is part of the triple-helical (collagenous) portions of the type IV basement membrane collagen.

Higher Molecular Weight Structures Containing the GP Antigen. As is discussed above, other components that react with serum from patients with GP are less retarded on gel chromatography than is the GP antigen—i.e., fractions S-200-I and S-200-II (Fig. 1). Such heterogeneity of the antigens in collagenase digests of GBM has previously been shown by Holdsworth et al. (28) and Hunt et al. (15). Antigens having apparent molecular weights of 27,000 and 53,000 (28) or 25,000 to 200,000 (15) were demonstrated.

The nature of the antigens present was further studied. Components in fraction S-200-I were not dissociated into
smaller structures by NaDodSO4 as shown by NaDodSO4/polyacrylamide gel electrophoresis followed by immunoblotting (data not shown). It is possible that this fraction mainly contains GP antigens only partially solubilized by the digestion with collagenase. Components in fraction S-200-II, on the other hand, could be dissociated both with NaDodSO4 and with 6 M guanidine-HCl to yield smaller molecules with a high activity in the GP antigen assay. Chromatography of this fraction (S-200-II) on Sephacryl S-200, eluted with 6 M guanidine-HCl (Fig. 4), gave two peaks containing antigen eluting at the same positions as the two peaks obtained when fraction S-200-III was chromatographed under identical conditions (cf. Fig. 2). NaDodSO4/polyacrylamide gel electrophoresis further verified that the fractions recovered from S-200-II also had apparent molecular weights of 48,000 and 26,000, respectively (Fig. 4). A portion of the material in peak I (indicated in Fig. 4) could be dissociated by reduction to yield the molecular weight 26,000 component (data not shown).

By using ELISA the similar character of the different antigens was further demonstrated. Antibodies present in serum from a patient with GP react with the antigens in pools I and II (cf. Fig. 4) and with the GP antigen (pool II; cf. Fig. 2). The binding was in all cases completely inhibited by the purified GP antigen—i.e., component II from fraction S-200-III (Fig. 5). Conversely, it was also shown that binding of antibodies to the GP antigen was inhibited by the other components (data not shown). It appears, then, that the components with apparent molecular weights of 48,000 as well as the components with apparent molecular weights of 26,000 contain identical antigenic determinants. It is possible that the larger molecules represent complexes of the GP antigen. Support for this hypothesis was provided by amino acid analysis of the molecular weight 48,000 component isolated from S-200-II (Table 1). Its composition is indeed similar to that of the purified GP antigen.

The molecular origin of the GP antigen is not disclosed by the present investigation. Its amino acid composition, however, is rather similar to that of the collagenase-resistant non-triple-helical NC1 portion of the type IV collagen. Furthermore, the size of the smallest component containing the GP antigen is rather similar to that of the NC1 (4). It is possible that the various aggregation forms of the GP antigen demonstrated may represent monomer, dimer, and trimer of the NC1 peptide portion of type IV collagen.

**Antibody Specificity of Different Patients.** Proteins in collagen-digested sera were separated by NaDodSO4/polyacrylamide gel electrophoresis and blotted onto nitrocellulose paper. Sera from seven patients with GP were incubated with strips of the paper. Subsequent labeling with anti-human IgG-peroxidase conjugate allowed demonstration of bound immunoglobulins from patients (Fig. 6). All patients gave the same patterns, showing strongest reactions with components with electrophoretic mobilities corresponding to molecular weights of 26,000 (i.e., the GP antigen) and 48,000.

### Table 1. Amino acid composition of GP antigen

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<th>Residue</th>
<th>M&lt;sub&gt;r&lt;/sub&gt; 26,000</th>
<th>M&lt;sub&gt;r&lt;/sub&gt; 48,000*</th>
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*Component I from S-200-II (Fig. 4).
In conclusion, patients with GP have circulating antibodies directed to one type of antigen, probably present in the basement membrane of monomeric as well as in aggregated forms. This antigen can also be demonstrated in basement membrane preparations from lung and placenta (unpublished observations). The antigen then provides an immunological marker of this disease, because patients with other forms of glomerulonephritis have no circulating antibodies to the GP antigen, whereas they may have antibodies to other GBM structures (16).

Skillful typing by Miss Margita Andersson is appreciated. Grants were obtained from the Medical Faculty of the University of Lund, the Swedish Medical Research Council (5739 and 6481), and the Riksförbundet för njursjuka.

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**Fig. 6.** IgG of sera from GP patients showing binding to the same proteins. Digests of GBM with collagenase were electrophoresed on NaDodSO₄/polyacrylamide gels. Proteins were blotted onto nitrocellulose paper and stained with serum from seven patients with GP, two control sera (NS), and one GP serum after adsorption of IgG with staphylococcal protein A-Sepharose (Protein A Ads). An eluate of the antibodies bound to the protein A-Sepharose was also used for staining (Protein A Eluate).


